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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

JAN 2 1975

Application For Research Grant

Date: 31 December 1974

1. Name of Investigator(s): (include Title and Degrees)
HANS MEIER, D.V.M., Dr. med. vet., M.R.S.H., Senior Staff Scientist
RICHARD R. FOX, Ph.D., Staff Scientist

2. Institution & Address:
The Jackson Laboratory, Bar Harbor, Maine 04609

3. Short Title of Project:
Oncogenesis in the rabbit: genetic susceptibility, vertical transmission of virus, and environmental influences.

4. Proposed Starting Date:
1 July 1975

5. Anticipated Duration of this Specific Study:
Three (3) years

6. Brief Description of Objectives or Specific Aims:

Hereditary lymphosarcoma and immune hemolytic anemia associated with thymoma in rabbits provide important new models for study of the pathogenesis of neoplasia, including probable viral oncogenesis, and immunopathological disorders. A search for and propagation of oncogenic RNA virus(es) or genomes in rabbits is important because of (a) the widespread distribution of these viruses among vertebrates, (b) their possible role as universal determinants of cancer, (c) our preliminary evidence for the presence of C-type RNA virus and polymerase in these rabbits, and (d) the many experimental uses of rabbits in biomedical research.

We have three strains of rabbits that have pathologies relevant to this study of the interaction of host genotype, environment, and C-type RNA virus(es): strain WH with its hereditary lymphosarcoma (1); strain X with its hereditary autoimmune hemolytic anemia associated with thymoma (2), and strain III an ahh inducible rabbit strain highly susceptible to tumorigenesis induced by ethylnitrosourea (3) (see CTR #951 renewal application).

We are attempting virus-isolation following established procedures for murine, avian, and feline leukemia viruses. It should be possible to sediment virus from rabbit tissue by ultra- and gradient centrifugation. Isolated and purified virus can then be used as antigen(s) for the production of viral specific antisera, both against coat proteins and the group-specific antigen. We also need additional information to decide whether the same gene, which is responsible for susceptibility to hereditary immune hemolytic anemia, also predisposes to thymoma; and whether both hemolytic anemia and thymoma are due to an interaction with a vertically transmitted (inherited) C-type RNA genome. Because strain WH and X are genetically related, a common hereditary basis is being sought for all three conditions. Lines susceptible and resistant to tumorigenesis may be obtained within a strain.

7. Give a Brief Statement of your Working Hypothesis:
Because our studies of the lymphosarcoma and hemolytic anemia are compatible with the concept of both a genetic susceptibility and vertical transmission of a virus, we hypothesize that the two phenotypes are the result of a specific viral-host interaction.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See attached.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

The Jackson Laboratory: Main Laboratory, Hamilton Laboratory, and Virus-Leukemia Laboratory.

10. Additional Requirements:

None.

11. [illegible]ophical sketches of all principal and professional personnel (append)

See attached.

12. List of publications: (Five most recent as pertinent) (append)

See attached.

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R: REDACTED MATERIAL

3.

13. Budget: (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Hans Meier, Project Director	5	REDACTED
R. R. Fox, Project Co-Director	15	
Technical		
D. D. Crary, Professional Assistant	10	
R. P. Norberg, Professional Assistant	20	
E. F. Farrin, Research Assistant	15	
Animal Caretaking		
Secretarial Assistance		
	Sub-Total	REDACTED
Employee benefits: 16% of Personnel		
B. Consumable Supplies (list by categories)		
Chemicals, glassware, instruments		2,100
	Sub-Total	$ 2,100
C. Other Expenses (itemize)		
Domestic travel to scientific meetings		300
Publication costs		300
Art, Photo, and Replicating Service		200
Food and bedding		2,300
	Sub-Total	$ 3,100
D. Permanent Equipment (itemize)		
None		0
	Sub-Total	$ 0
E. Overhead (15% of A+B+C)		$ 3,257
	Total	$24,973

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	$2,250	$3,350	—	$3,565	$27,333
Year 3	REDACTED	$2,550	$3,650	—	$3,928	$30,112

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

HANS MEIER
Signature
Director of Project (207) 288-3373, X 273 Telephone

AUSTIN C. CARTER
Signature
Business Officer of the Institution
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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Title of Project	Source	Amount	Duration
Current			
Oncogenesis in the rabbit: genetic susceptibility, vertical transmission of virus, and environmental influences. (#758)	The Council for Tobacco Research, U.S.A.	$ 24,401	7/1/74-6/31/75
Natural occurrence of RNA tumor virus (genomes) and host--gene control of their expressions.	National Cancer Institute	438,496	5/1/74-4/30/75
Hereditary diseases.	National Eye Institute	27,276*	6/1/74-5/31/75
Rabbit inbred and mutant stocks resource.	NIH Division of Research Resources	85,515	1/1/74-12/31/74
Transplacental effects of nitrosocompounds in inbred strains of mice and rabbits. (#951)	The Council for Tobacco Research, U.S.A.	25,461	7/1/74-6/30/75
Pending			
Transplacental effects of nitrosocompounds in inbred strains of mice and rabbits.	The Council for Tobacco Research, U.S.A.	27,010	7/1/75-6/30/76
Hereditary diseases.	National Eye Institute	24,698	6/1/75-5/30/76
Rabbit inbred and mutant stocks resource.	NIH Division of Research Resources	40,000*	1/1/75-12/31/75

*Direct costs

BUDGET JUSTIFICATION

The studies proposed in this application take advantage of the availability of several strains of rabbits and mutant stocks at the Jackson Laboratory (see appendix). We expect them to prove of considerable value in studies of tobacco products.

Although a wide variety of spontaneous infectious and hereditary diseases have been found in the rabbit, tumors have been reported infrequently. However, only a few systematic studies have been conducted. Our studies reveal that lymphosarcomas and hemolytic anemia occur with high frequency in rabbits, but that the incidence, type, and development are greatly influenced by age, breed, and other constitutional factors. Clearly, studies with genetically controlled rabbits both supplement and complement studies with inbred mice. This unique resource of rabbits at the Jackson Laboratory must be <u>maintained</u> and made <u>available</u> to research workers elsewhere. The two strains of rabbits, WH and X, are extremely valuable for studies in oncogenesis but their exploitation has hardly begun. Strain III has an excellent research potential because of its aryl hydrocarbon hydroxylase (AHH)-inducibility. Fortunately, we have the professional staff and talent essential to the studies that we propose. The financial support requested from The Council for Tobacco Research for maintenance and study of these rabbits is minimal, but is adequate when coupled with existing support.

There is no need, at present, to include in this budget salary provision for 100% effort contributed by the project co-directors because this proposal relates to work supported by NIH research contract N01 CP 33255 from the National Cancer Institute and NIH resource grant RR 00251 from the Division of Research Resources of NIH.

8. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURE

Introduction and background

We stated in detail the aims of our studies in our applications (1970-1974) for Research Grant #758. Clearly, the work we proposed, particularly the viral studies, could not be completed within the period for which we received funding; at least 3 additional years of work and support are required. Thus, this application is for continuance of studies currently underway.

The overall goals of our studies remain basically as proposed previously:

1. We have observed within a few years over 93 cases of lymphosarcoma in a small breeding colony of strain WH rabbits, and affected animals of both sexes were found in each of several generations. Because of the unusual case aggregation of lymphosarcoma, we wish to investigate the host genetic factors conferring susceptibility to lymphosarcoma, the mode of inheritance or transmission, the probability of a vertically transmitted virus, and the environmental influences that may modify incidence and pathogenesis of lymphosarcoma.

2. Another strain of rabbits, strain X, which is genetically related to the WH strain, is characterized by a high incidence of immune hemolytic anemia (76 cases); and thymoma occurs as well. We want to find out the mode of inheritance or transmission of immune hemolytic anemia and thymoma in strain X rabbits; then, because the two strains are genetically related, we can evaluate the possibility of a common hereditary basis for all conditions in both strain X and WH. The various clinical or phenotypic expressions probably derive from differences in the genetic background of the two strains.

3. We believe that all three conditions are caused by a vertically transmitted virus analogous to the C-type RNA viruses occurring in a number of vertebrates, including man. The outcome of viral-host interaction depends to major degrees on host genetic factors, but it may be modified by environmental influences.

Specific aims

Studies of the interaction of host genotype, environment, and virus, if present, are complex and some narrowing of aims is necessary. Thus, the specific aims are:

1. To isolate and propagate a C-type RNA virus from rabbits,

2. To study its biological, biochemical, and biophysical properties, and

3. To relate its function, if any, to lymphosarcoma, hemolytic anemia, and thymoma.

Scientific progress during tenure of current grant

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A brief background and resume of work accomplished thus far is pertinent to the studies proposed in this application. Detailed reports of our accomplishments have been submitted (see previous progress reports).

1. Assays and distribution of DNA-polymerases in rabbit tissues. RNA tumor virus(es) contain an enzyme, RNA-dependent DNA polymerase, that transcribes DNA from an RNA template. The role of this enzyme has not as yet been established,

i.e., whether or not it plays a part in transformation or may be essential for a transformed state.

Introduction and background

In collaboration with Dr. Masa Hatanaka and Dr. Gilden of Flow Laboratories, we assayed organs of normal, azathioprine (Imuran)-treated, and lymphosarcoma-afflicted rabbits. Most organs, both normal and malignant, revealed enzyme activity. Thymuses, major mesenteric and popliteal lymph nodes, and gastrointestinal tract have the highest specific activity of RNA-dependent DNA polymerase suggesting the common nature or origin of the lymphopoietic systems. Also, the data taken together with our serological findings (previously reported) verify the presence of an RNA viral genome in WH rabbits. It is also present in other incipient inbred strains of rabbits, i.e., III_{DW} and a hybrid between two strains $(III_{mo} \times III_{c})\ F_1$.

2. Natural occurrence of RNA tumor viruses. Our earlier findings of interspecies group-specific antigen or gs-AG (gs-3) reactivity indicates that a type-C RNA viral genome must be present in the rabbit (4). Since complete C particles are absent, the genome must largely occur in covert or incomplete form. However, there may be sites of predilection, e.g., bone marrow, progestational uterus, blastocyst, uterine secretions, etc., where complete infectious virus is expressed.

In the light of findings of C-type particles in early mouse embryos (5), we considered the possibility that the progestational and estrus uterus, blastocyst, and uterine secretions of various rabbit strains may harbor C-type particles. This approach coincides with that of Daniels (6) who accidently observed C-type particles in rabbit blastocysts in studies designed to evaluate the role of the uterus in "providing information" for the growth and differentiation of the embryo. Our preliminary findings were discussed in detail by Dr. Meier at the SAB meeting in Arizona in the spring of 1974.

3. Pedigree analyses for lymphosarcoma and immune hemolytic anemia susceptibilities. We have now observed 93 cases of lymphosarcoma in strain WH and seven cases in genetically related rabbits of strain AX. Autoimmune hemolytic anemia occurred in 76 rabbits of strain X; in addition, seven cases were found in strain AC which is in part derived from strain X. In fact, all affected individuals in the four strains are genetically related and trace back to a common ancestor, X974. Thus, we suspect that the two different syndromes, each caused by an autosomal recessive gene, ls and ha, respectively, may indeed be manifestations of the same gene with the phenotype dependent upon the remainder of the host genotype. To either confirm or rule out this possibility, we are now awaiting results of sufficient matings between ls/+ and ha/+ heterozygotes.

Certain of these aspects including our pathological and hematological findings in strains WH and X afflicted with lymphosarcoma and immune hemolytic anemia have been presented at the First Annual Veterinary Symposium of Hycel, Inc., (7) and in two book chapters (8, 9).

4. Myeloid leukemia in strain III. We have reported the first case of myeloid leukemia in the rabbit. It occurred in a 13-1/2-month-old male of subline III_{ep}. Its features are distinct from hereditary lymphosarcoma by cell type, organ involvement, and distribution of tumors. Studies are in progress to determine whether this case of myeloid leukemia is of hereditary origin. Also, we plan to determine in additional cases whether an oncogenic type-C RNA viral genome is involved in this tumor, as well as in lymphosarcoma of WH rabbits (10).

5. Genetic predisposition to tumors in the rabbit. Our analysis of genetic

factors has been presented in Progress Report 2, and a paper describing our findings has been published (11).

6. Hereditary hemolytic anemia associated with thymoma in strain X rabbits. Autoimmune (Coombs-positive) hemolytic anemia occurs with high frequency in rabbits of strain X. This condition is rapidly fatal with a mean survival time of about 5 months. Sometimes it is associated with thymic hyperplasia and thymoma. The gene conferring susceptibility, designated ha, may be identical with that causing lymphosarcoma susceptibility and assigned the gene symbol, ls, in WH rabbits. Strains X and WH are closely related genetically, and a common gene responsible for all conditions may have phenotypic expressions that are dependent upon the remainder of the genotype. We are also considering the possibility that a vertically transmitted virus similar or analogous to the C-type RNA virus of mice is of etiological importance in addition to gene(s) conferring susceptibility (2).

Methods and procedures of proposed studies

In our studies of the pathogenesis of lymphosarcoma in WH rabbits and of hemolytic anemia associated with thymoma in strain X rabbits, we wish to achieve an understanding of the underlying mechanisms leading to each disorder in the two strains. No doubt the interactions of host genotype, "environment," and C-type RNA virus, if present, are complex. In the light of our findings to date we are proposing the following studies:

1. Characterization of viral protein markers. It appears that the C-type virus markers, group-specific antigen (gs-AG) and RNA-directed DNA polymerase, are present in rabbit tissues. We propose to study the chromatographic properties, template and cation preferences, and the sedimentation coefficient of the rabbit "viral" polymerase as well as its immunological relationship to known viral polymerases.

We found peaks of DNA polymerase activity, which correspond with MLV polymerase, when fractions from 10 to 30% glycerol gradients were surveyed with the template primer poly rA.oligo dT, whereas no peaks were detected with $(dT)_{12-18}$ alone (Fig. 1 and 2). The absence of activity in the presence of $(dT)_{12-18}$ rules out the possibility of contamination with terminal deoxyribonucleotidyl transferase. Polymerase activity was 10 times higher in the gestational uterine tissue than in the nongestational rabbit. A shift in peak positions occurred which may be due to the presence of stimulators and inhibitors from an impure preparation. Further purification by sephadex-chromatography, use of other template primers, and analysis of the immunological relationship between the rabbit polymerase and other known viral polymerases are necessary for the ultimate characterization of this enzyme.

Further support for the presence of C-type RNA virus and polymerase in rabbits is given by banding patterns (12) and electron microscopy (13). Figure 3 demonstrates that uterine fluid labelled with ^{3}H-uridine 24 hours before banding contains virus polymerase with a bouyant density of approximately 1.15 g/ml. Extensive electron microscopic studies of 5-day blastocysts and uterine fluid revealed the presence of budding type-C particles.

Extracts from the uterus and from other rabbit tissues (lymphosarcoma and normal tissues) will be passed over a microgranular DEAE-cellulose column (14). The viral reverse transcriptase, if present, and the cellular polymerase (DNA polymerase II) elute from the column at low salt concentrations and are thus separated from other cellular DNA polymerases (DNA polymerase I and III) which

Fig. 1. Reverse transcriptase activity from uterine tissue of 14-day pregnant strain III_{VO} rabbit.

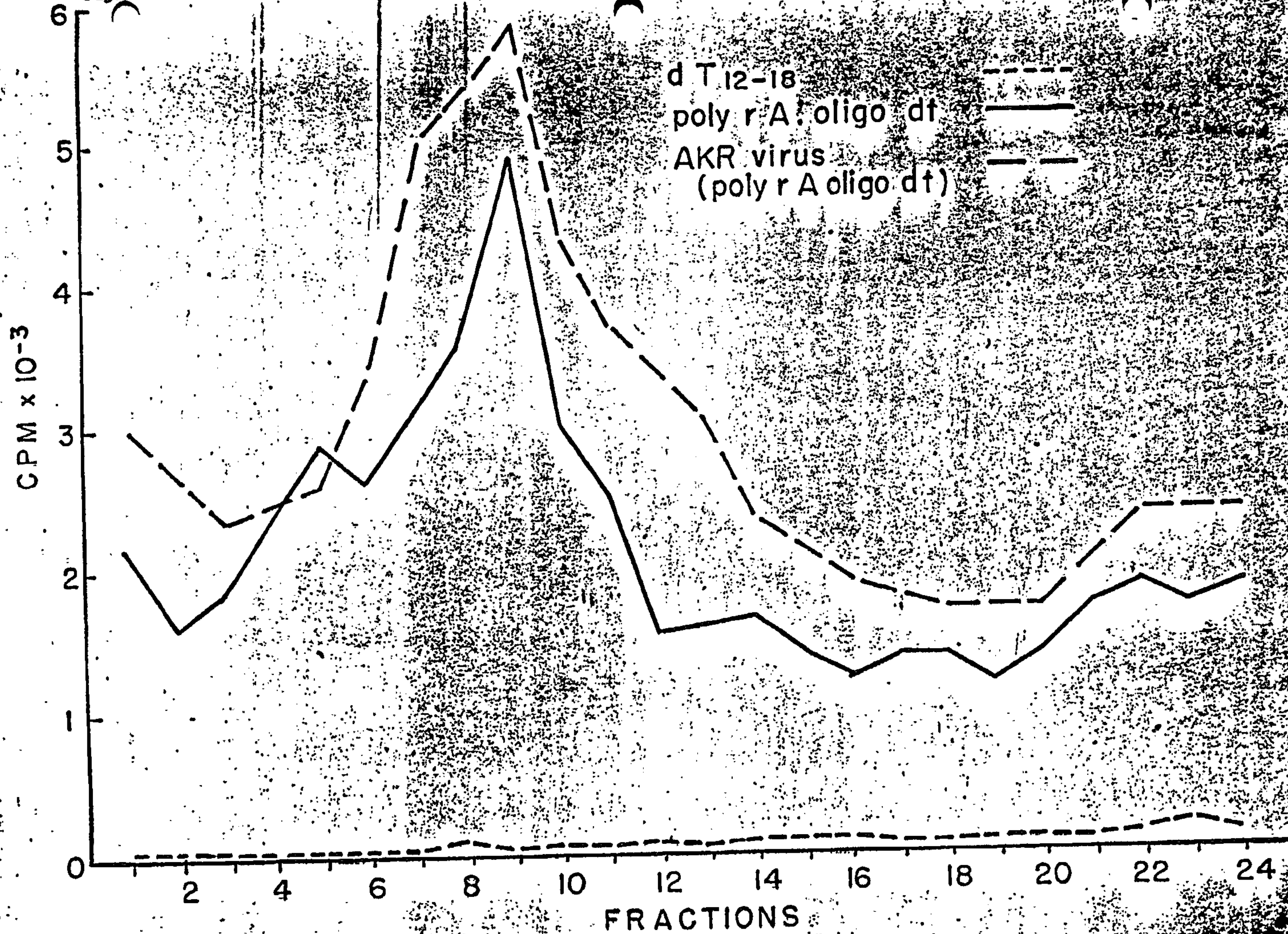

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Fig. 2. Reverse transcriptase activity from uterine tissue of induced hydrouterus.

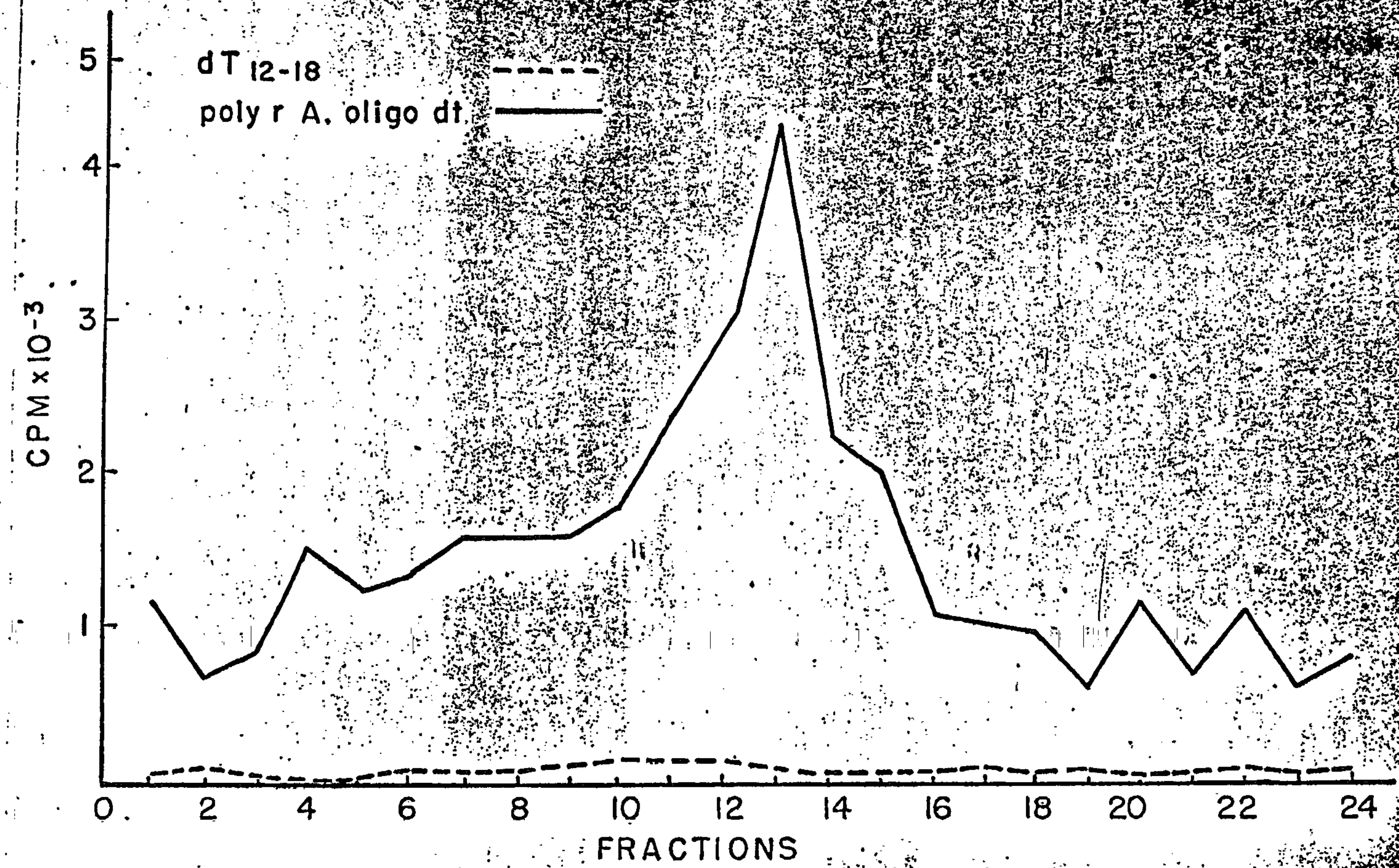

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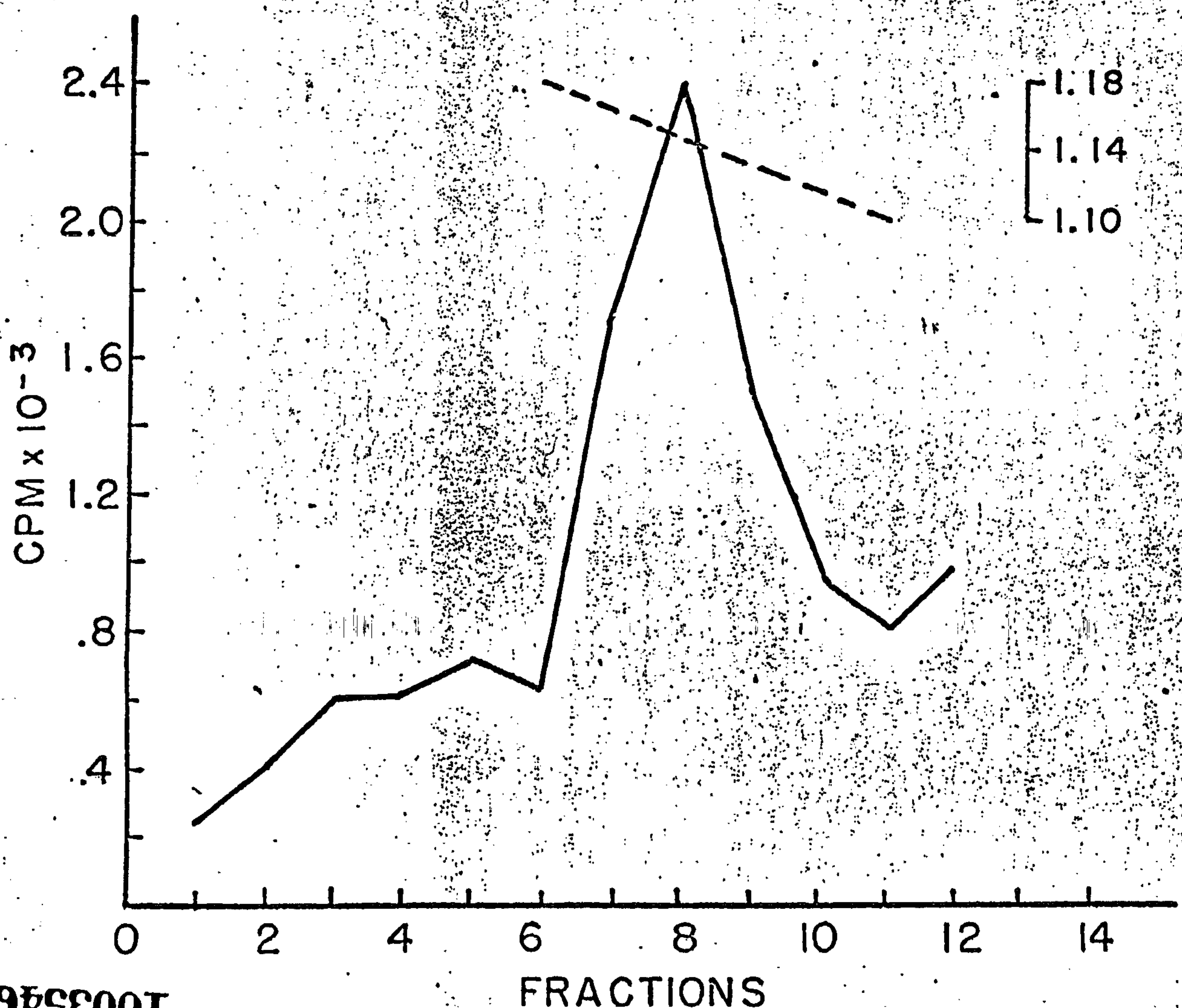
Fig. 3. Reverse transcriptase activity of uterine fluid from induced hydrouterus.



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elute at a higher ionic strength (14). The peak fractions, determined by absorbance at 260/280, from the phosphocellulose column eluting at 0.26 M KCl are then pooled and concentrated by dialysis against a polyethylene glycol buffer.

2. DNA polymerase assays. The DNA polymerase assays will be as described by Lewis (14) using various templates and concentrations of Mn^{2+} or Mg^{2+} as follows:

Template primer	Tritium-labeled substrate	Divalent cation
$(A)n.(dT)_{12-18}$	TTP	Mn^{2+} or Mg^{2+}
$(C)n.(dG)_{12-18}$	dGTP	Mn^{2+} or Mg^{2+}
$(dA)n.(dT)_{12-18}$	TTP	Mn^{2+} or Mg^{2+}
$(dT)_{12-18}$	TTP	Mn^{2+} or Mg^{2+}
$(dG)_{12-18}$	dGTP	Mn^{2+} or Mg^{2+}

3. Velocity gradient sedimentation. Samples of enzymes will be layered onto 5 to 20% sucrose gradients and centrifuged for 16 hours at 150,000 x g at 4°C in a Spinco 50.1 rotor. Fractions will be collected by bottom puncture and analyzed for DNA polymerase activity. Protein markers will be processed similarly on gradients and detected by absorbance at 280 nm (15).

4. Antibody inhibition studies. Antibody inhibition studies will be performed as described by Todaro (12). Antibody to the rabbit "viral" polymerase will be prepared in rats (16). A portion of the viral polymerase is incubated with an equal volume of antibody to known viral polymerase. A DNA polymerase assay is then performed to measure residual enzyme activity.

5. Attempts at isolation of rabbit C-type RNA virus. In the light of findings in inbred strains of mice, we shall consider the possibility that rabbits may harbor two types of viruses, ecotropic and xenotropic, as discussed below. Thus, our approaches to identifying these viruses include EM studies, cocultivations, RT assays (including simultaneous detection methods for both RT and 70S RNA), isopycnic zonal banding, mixed lymphocyte reactions (MLR in vitro), and graft-versus-host reaction (GvH) in vivo. Details of some of these approaches are described below; others have been described previously:

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Our searches for ecotropic rabbit type-C RNA virus(es) will include tissue EM, RT assays, radioimmunoassay, hybridization reactions, and, isopycnic zonal banding of rabbit tissue culture (TC) supernates and uterine fluids.

a. Electron microscopic studies. We shall primarily focus on the pro-

gestational and estrus uterus, blastocysts, and pellets of uterine secretions. Virus particles have previously been found in mammalian embryos of several species and stages, particularly the mouse (13). Small A-type particles were common in early stages but absent in blastocysts, but C-type particles occurred only in blastocysts (5).

Tissues are fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer and then postfixed in 1% osmic acid. Postfixation is followed by dehydration in ethanol and embedded in Epon-Araldide (17). Ultra thin sections are cut on a Porter Blum I microtome and stained with uranyl acetate and lead citrate. Tissues will be examined for C-type particles in a Hitachi HU-11 C electron microscope.

b. Reverse transcriptase assays and isopycnic zonal banding. Uterine secretions and fluids of non-cocultivated rabbit cell cultures will be assayed for RT by a modified technique of Ross et al. (18). Uterine secretions and fluids from both progestational and estrus stages will be collected from rabbits following surgical cervix ligation. They will then be assayed for RT directly (18), pelleted, and pretreated for isopycnic banding (19). RT determinations are particularly useful for assay of low-titered viral preparations or with suitable viral infectivity assays lacking.

c. Simultaneous detection assay. The development of the "simultaneous detection assay" (SDA) directly provides evidence for the concurrent analysis of two unique characteristics of C-type RNA viruses, namely, a 70S viral RNA associated with an RNA-dependent DNA polymerase (20). In a reaction mediated by a C-type virus, ^{3}H-DNA will sediment in a 70S region of the gradient representing the 70S RNA:^{3}H-DNA reaction product. The successful use of the SDA to detect RNA viruses in mouse and human milk (20) may also be applicable for the detection of this reaction product in rabbits.

Uterine fluid and tissues from rabbits with lymphosarcoma will be examined for the presence of 70S RNA:^{3}H-DNA as described by Schlom and Spiegelman (20). The sensitivity of the assay makes it a useful tool for detecting the presence of C-type virus in rabbit tissues.

Biophysical properties can be utilized to indicate the presence of viral agents in culture (21). The RNA tumor viruses band at a density of 1.16 to 1.18 g/ml in a continuous sucrose gradient (15% to 60%).

Rabbit cell cultures are injected with labelled uridine and the fluid is collected 24 hours later; alternatively, growth medium containing 20 uCi/ml of ^{3}H-uridine are added to subconfluent cultures and incubated for 24 hours at 37°C. Supernates or fluids are then examined as described by Panen and Kirstein (21), and Kruse and Patterson (22).

d. Purification of group-specific (gs) antigen. High speed virus pellets from culture fluids are disrupted and gs-antigen (P30) purified by phosphocellulose chromatography and pressure dialysis using the same procedures for the purification of RT and described by Ross and Scolnick (18) and Scolnick et al. (23).

e. Radioimmunoassay of gs-antigen. Interspecies (gs-3) antigen is determined by a competitive radioimmunoassay (16) using ^{125}I Rauscher murine leukemia virus gs-antigen purified by gel filtration and isoelectric focusing (23). Protein is determined by the method of Lowry et al. (24).

f. Extraction of cellular DNA and RNA. Rabbit cells are suspended in three volumes of 0.05 M Tris-HCl, pH 8.3, 5 mM magnesium acetate, and 0.04 M sodium chloride, homogenized, and centrifuged at 10,000 x g. The pellet is resuspended in 20 volumes of the buffer adjusted to 1% sodium dodecyl sulfate, and extracted at room temperature with chloroform-isoamyl alcohol (24:1 V/V) and with neutralized water-saturated phenol containing 10% m-cresol. After phenol extraction, the solutions are extracted four times with ether to remove the phenol and treated with 0.5 N KOH at 49°C for 12 to 16 hours to hydrolyze RNA. The remaining DNA is neutralized and dialyzed against three changes of 300 volumes of 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 10^{-4} M EDTA, and stored at -20°C at a concentration of approximately 8 mg/ml. One gram (wet weight) of cells should yield about 2 to 5 mg of DNA. Analysis of the optical density profile of this DNA on Cs_2SO_4 gradients are not expected to reveal RNA in the preparations. Cellular RNA is extracted as previously described by Benveniste et al. (25).

g. Synthesis and purification of viral ^{3}H-DNA. The endogenous reverse transcriptase reaction from detergent-disrupted rabbit type-C virus is used to synthesis (^{3}H)thymidine-labeled DNA in the presence of actinomycin D (50 ug/ml) as described previously by Benveniste and Scolnick (26). The specific activity of the ^{3}H-DNA is 2.0×10^7 CPM/ug.

h. Hybridization reactions. Approximately 2000 counts/min (0.1 ng) of enzymatically synthesized DNA is incubated with either cytoplasmic RNA or with DNA in 10^4- to 10^7-fold excess for 48 to 72 hours at 31°C in 0.20 ml reaction mixtures containing 0.015 M Tris-HCl, pH 7.4; 0.15 M sodium chloride; 5×10^{-4} M EDTA; 0.1% SDS, and 38% formamide. The extent of hybrid formation can be detected by hydrolysis with purified S_1 nuclease as described previously by Benveniste and Scolnick (26) and Benveniste et al. (25).

Our searches for xenotropic rabbit type-C RNA virus(es) will consist of MLR, graft-versus-host reaction (GvH), RT, cocultivations, and focus assays.

a. Mixed lymphocyte culture reactions (MLR) and graft-versus-host (GvH). The evidence that viral oncogenesis can be enhanced by immunosuppression is now overwhelming (27). A combined electron microscope and virologic analysis by Schwartz et al. (28) showed that MLV could be activated during the GvH and MLC reaction in mice. A similar procedure will be followed in rabbits using (III x WH)F_1 with one of the parents as the donor of lymphocytes. In the GvH each F_1 will receive four intraperitoneal injections of 60×10^7 cells once a week for 4 weeks. The F_1 rabbits will be killed 10 days after the last injection. Spleens will be taken for EM, culturing, and polymerase assay. Control rabbits will include: (1) Normal F_1 rabbits with no treatment; (2) F_1 rabbits injected with adjuvant; (3) F_1 rabbits given sheep red blood cells; (4) F_1 rabbits given allogenic spleen cells; and (5) F_1 rabbits given parental spleen cells which have been treated in vitro with mitomycin C before injection. Each control group will receive treatment once a week for 4 weeks.

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The counterpart in vitro of the GvH reaction, the MLC reaction, will be examined for the induction of C-type virus in the rabbit. Lymphocyte suspensions will be prepared from spleens of young rabbits [strain III, WH, and (WH x III)F_1]. Spleens will be minced, passed through progressively smaller syringes, and put through a Ficoll-gradient as described by Kruse and Patterson (22) (bone marrow). For the MLC reaction, 2.5×10^6 cells per ml of either strain III or WH will be incubated with 2.5×10^6 cells per ml F_1 lymphocytes in sealed test tubes in volumes of 3 ml for 6 days. The cultures will be supplemented with 1 ml of RPMI 1640 at 48-hour intervals and assessed for cell proliferation by the addition of 1 uCi

of ^{3}H-thymidine to 1 ml of the cultures 4 hours before the termination of the experiments in a liquid scintillation counter. Cell proliferation is assessed by precipitation with trichloracetic acid. Control cultures include (a) strain III or WH and F_1 lymphocytes incubated alone and (b) F_1 lymphocytes incubated with mitomycin treated strain III or WH lymphocytes. Supernatants from all cultures will be assayed for virus production by the reverse transcriptase reaction.

In inbred strains of mice, GvH occurs across the major histocompatibility (H-2) region (29). However, analysis of a large number of H-2 crossovers and their parental strains revealed that the strongest GvH reaction was associated with the Ir (immune response)-region (29). Apparently, the products of these genes are receptors on the surface of thymus-derived lymphocytes (T-cells). Histocompatibility loci exist as well in the rabbit; the major locus, RL-A, is similar to the Ag-B of rats and H-2 of mice (30). Thus, it is likely that Ir genes are present also, and we may take advantage of Ir differences in attempts to activate endogenous type-C virus. Both eco- and X-tropic viruses have been activated in mice by MLV and GvH.

b. Cocultivation and focus formation. Tissues for cell cultures will be removed asceptically from various organs of normal and lymphosarcomatous rabbits of different strains, WH, X, III, etc; cultures will be established according to standard procedures (22) and subcultured after confluency using 0.1% trypsin in Hank's balanced salt solution.

The rabbit-cell cultures as well as those cells used for cocultivation are maintained in plastic (Falcon) flasks containing Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 0.3% glutamine, and gentomycin (0.6 ml/100 ml medium). Cells used for cocultivation are non-virus-yielding newborn rat kidney cells transformed by the Harvey strain of murine sarcoma virus (NRK-Harvey or H-NRK), human rhabdomyosarcoma (RD), and human embryo skin-muscle fibroblasts (HESM), NRK, and various BALB/c virus (MuLV)-negative cell lines (A31, R4, and S16). Their use and derivation has been amply documented (31-34). The cocultivation procedures are those described by Levy (35). Briefly, we shall follow these lines:

Direct cocultivation of 4×10^5 rabbit cells with 1×10^4 Harvey virus transformed newborn rat kidney cells (H-NRK); the culture fluid will be changed every 2 to 3 days and the 7-day fluid collected.

An alternate procedure consists of cocultivating rabbit cells with rhabdomyosarcoma cells (RD), human embryonic skin and muscle cells (HESM), and BALB/c cells separately, following pretreatment with DEAE-dextran (25 ug/ml). After 7 days the respective cultures will be trypsinized and split. One dish will be cocultivated with the H-NRK, whereas we shall grow the second dish for another week before cocultivation.

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The 7-day cocultivation fluids will then be assayed for RT and focus formation on normal rat (NRK), human (RD, HESM), mouse (BALB/c-A31, R4, and S16), and rabbit embryo cultures as well as for gs-antigen and hybridization reactions as previously described. Xenotropic viruses would be expected to grow on sensitive foreign host (heterologous) cells but not homologous cells (35).

6. Studies in strain X rabbits. Information is needed to decide whether (a) the same gene that is responsible for susceptibility to hereditary immune hemolytic anemia also predisposes to thymoma, (b) the gene giving rise to hemolytic anemia or thymoma, or both, in strain X is the same gene that is responsible for lymphosarcoma-susceptibility in WH rabbits, and (c) both hemolytic anemia and thymoma are due to an interaction with a vertically transmitted C-type RNA viral genome.

The following procedures should provide the answers sought to the first two questions. Information on the third will result from approaches identical or analogous to those described for WH rabbits.

a. Is the same gene responsible for both hemolytic anemia and thymomagenesis? We are chronically treating strain X rabbits homozygous for the hemolytic anemia trait (ha/ha) with the immunosuppressive drug, azathioprine (Imuran). Azathioprine should prevent its occurrence because the hemolytic disease is due to an immune disorder. However, by analogy to NZB mice, it should not interfere with development of thymoma. Thus, in order to establish that the gene is responsible for both conditions, we must be able to selectively induce thymoma upon continued azathioprine therapy.

b. Are the genes, lymphosarcoma-susceptibility (ls) and hemolytic anemia (ha), identical? In order to answer the question of allelism of ha and ls, F_1 hybrids between respective heterozygotes (ls/+ and ha/+) should yield approximately 25% abnormal offspring if the two genes are allelic. We cannot of course decide a priori whether they resemble either of the respective homozygotes (ls/ls and ha/ha) or whether they have clinical features of both.

Genetic studies are time-consuming and require large numbers of animals. Also, we cannot know a priori what the latency period may be for clinical signs to appear if heterozygotes (ls/ha) are obtained.

In past and current breeding experiments we have made the following crosses, ha/ha x ls/+ and ha/+ x ls/+; indeed, several presumptive heterozygotes (ls/ha) have been observed. These findings clearly indicate the identity of ha and ls. In order to obtain a representative spectrum of phenotypic expression of compound heterozygotes, we anticipate a requirement of at least 10 afflicted rabbits.

So far, affected rabbits suffered either from hemolytic anemia or a combination of hemolytic anemia and a lymphoproliferative disorder depending upon the age at which clinical signs appeared. Although we now have nine afflicted progeny (compound heterozygotes), a number of potentially affected rabbits are still alive.

7. Both strains of rabbits. Strains WH and X are partially inbred. The coefficients of inbreeding, which defines that proportion of loci for which the original or base population was heterozygous but which through inbreeding has become homozygous, are approximately 0.72 and 0.88, respectively. We estimate, therefore, that each strain may be homozygous for as many as 80% of the initially variable loci and that these loci have been fixed for different alleles in the two strains because of deliberate selection for and maintenance of specific mutant genes.

A population of animals need not be inbred for an analysis of the inheritance of a specific gene or phenotypic trait. The genetic basis of a trait by which two strains differ can be obtained by crosses to obtain F_1, F_2, and backcross generations. From a population segregating in Mendelian proportions for recognizable phenotypes, one may estimate the number of gene pairs that distinguish the two parental strains with respect to the trait in question. Conversely, because there is reduced genetic variability within inbred strains, they are unsuitable for selection experiments unless genetically heterogeneous populations are synthesized by crossing two or more strains. Because the WH and X strains are now only partially inbred, we can select for those genes that produce a particular phenotype of interest, e.g., lymphosarcoma susceptibility and resistance. Thus, in each strain we should be able to produce two or more lines that may vary in tumor development and also produce a line that is tumor-resistant. These may interact specifically, but variously in studies of chemical or other cocarcinogenesis.

In addition to the 93 cases of lymphosarcoma in strain WH, we have now also found seven cases in genetically related rabbits of strain AX. Also, in addition to the 76 cases of autoimmune hemolytic anemia in strain X, seven cases were observed in strain AC, which is in part derived from strain X. In fact, all affected individuals in all four strains are genetically related and trace back to a common ancestor, X974.

We are now looking for circulating antinuclear and anti-DNA antibodies in the various rabbit strains, as well as immune complex disease in biopsies from kidneys. Because lymphoid cell lines maintained in suspension on a gyratory shaker have yielded complete virus from NZB and related mice, we are using analogous conditions for cell lines produced from spleens and lymph nodes.

Significance of this research

The proposed studies relate to an opportunity for the analysis of two major groups of disorders: cancer and autoimmune disease. They do not deal directly with the effects of tobacco, but are clearly relevant to tobacco effects in several indirect ways: (a) as neither tobacco nor its various chemically defined components nor all known carcinogenic chemicals provide a host with the genetic information to produce or induce cancer and any other disease, (b) the occurrence of cancer or any non-neoplastic disease is dependent upon the inborn host-genetic regulation of all processes allowing or disallowing it to occur. Thus, an analysis of the hereditary pathways and their acquired modifications through tobacco or other means is fundamental to an understanding of all disease processes. Some of these may be attached by use and study of the two mutants (ls/ls and ha/ha) of rabbits. Because of their analogies, they may help clarify mechanisms of the respective human disorders and provide basic information about their pathogenesis.

So far, we have made three major findings: (a) the likely identity of the genes conferring susceptibility to both cancer (lymphosarcoma) and immune hemolytic anemia (autoimmune disease), (b) the most probable presence in WH, X, and other rabbit strains of an endogenous oncogenic C-type RNA tumor viral genome, and (c) strains WH

and X have Coombs' autoantibodies. The major significance of our studies lies in the potential relationship of these three observations.

1. Overall significance. Like human systemic lupus erythematosus (SLE) and the related disease in New Zealand Black (NZB) mice and (NZB x NZW)F_1 hybrid mice, hereditary autoimmune disease in strain X rabbits is associated with synthesis of various autoantibodies to erythrocytes and various nuclear materials (36). Some of the possibilities for their occurrence are that: (a) The hosts are unable to repair nucleic acids properly, and altered nucleic acids might either have an abnormal catabolic fate or antigenicity. This situation would be analogous to the defect in the repair of DNA that occurs in xeroderma pigmentosum. (b) The hosts are deficient in or have abnormal nucleases, and perhaps other catabolic enzymes, that result in the formation of immunogenic breakdown products from spent, autologous cells. (c) The hosts have a defect in some intracellular structure such as the nuclear or plasma membrane resulting in an inability to keep nucleic acids in a proper configuration or an intracellular compartment. (d) The hosts harbor a virus whose genome is immunogenic because of a different configuration or nucleotide composition from that of the host, thereby terminating tolerance to autologous nucleic acids (Meier, unpublished).

Thus, it seems possible that cells from patients with SLE or from diseased NZB mice are variants of one or more of the defects listed. Their detection and evaluation is clearly important in elucidating the pathogenesis of SLE, as well as the catabolism, structure, and repair of nucleic acids in normal cells.

In addition to autoantibodies to double-helical DNA, DNA-histone complexes, single-stranded DNA, and nucleolar RNA and their complexes, NZB mice also make antibodies to the endogenous C-type RNA tumor virus (36). The presence of such a genome in both strains WH and X, as well as all other rabbit strains is most probable. Thus, with genes ls and ha being identical and conferring susceptibility to either lymphosarcoma or autoimmune hemolytic anemia, a common pathogenesis of the two disorders is likely.

We now have evidence for a highly significant association in mice between the endogenous C-type RNA viral genome and tumorigenesis (37). In fact, viral expression in early life is a highly predictable marker for tumorigenesis with advancing age. This expression is host-gene controlled, and relates to tumors of all types, i.e., mesenchymal as well as epithelial tumors, and leukemias as well as solid tumors (37). Thus, the mechanism for tumorigenesis is hereditary or "built-in," but whether or not tumors will develop depends upon other host-genes as well as environmental factors (37). Although this explanation requires ultimate substantiation, it provides the most rational basis for all available tumor data.

2. Specific significance of project. Rabbits are of considerable value in biomedical research because of the vast amount of morphologic, physiologic, genetic, and biochemical data available, the simplicity of their care and breeding, and their large size. The finding of lymphosarcoma in the rabbit and its hereditary basis provides a new and important model for studies of the pathogenesis of neoplasia (1). The rabbit colonies at the Jackson Laboratory are free of Shope papilloma and fibroma, and myxomatosis viruses. Except for a small number of epithelial tumors, which have been described (11, 38), most tumors in our rabbits have been of lymphoid tissue origin, i.e., lymphosarcoma and thymoma.

Affected WH rabbits usually die between the age of 5 and 13 months. The

neoplastic involvement of lymphoreticular and other organs, especially kidneys, corresponded to a pattern observed in lymphosarcoma of other domestic animals (4). Specifically, it resembled in many ways visceral lymphosarcomatosis of cats that has been proved unequivocally to be caused by feline leukemia virus (1, 4). Similarities between rabbit and cat lymphosarcomas include the sites of onset, distribution of the neoplastic lesions, and the finding of a predominantly aleukemic hemogram (1, 4). However, in rabbit lymphosarcoma, we often found a relative increase in lymphoid cells including both immature and atypical forms (1).

If a C-type RNA virus is demonstrated, the ls and ha genes may confer susceptibility to malignant transformation of lymphoreticular tissues. We have found a number of genes in mice that enhance oncogenesis, especially leukemogenesis, but that do not influence the presence or absence of either murine leukemia virus (MuLV) or MuLV antigens (39).

If complete virus is inducible or is spontaneously expressed in any strain of rabbits, its isolation and purification is essential for the production of specific antisera. Also, yet another species may be demonstrated to harbor type-C RNA tumor viruses.

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Honors:	Royal Society of Health (Pharmacology) M.R.S.H.	1963
	Member, Scientific Advisory Board, The Council for Tobacco Research, USA	1971 -
	Member, Solid Tumor-Virus Working Group, Special Virus-Cancer Program, National Cancer Institute, NIH	1971 - 1973
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1955 - 1957	Harvard Medical School, Course Work in pathology and neuro-pathology
1957	Harvard University, Summer School of Arts and Sciences, and of Education (Biophysics and radioactive measurements)
1958	Armed Forces Institute of Pathology (Course in histochemistry)
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APPENDIX 1: CHARACTERISTICS OF RABBIT STRAINS AT THE JACKSON LABORATORY AS OF 15 OCTOBER 1974.

STRAIN (Name & Symbol)	COLOR	PREDOMINANT GENOTYPE	ORIGIN	COEFFICIENT OF INBREEDING(1)	PREDOMINANT ALLOTYPE	MATURE WEIGHT (GM)	OTHER GENES SEGREGATING
III (sublines)	Albino	cc AA E^dE^d as as	New Zealand	0.93	$a^1a^1b^4b^4c^7c^7$	3800	l, scoliosis(2)
(III)			White Castle '32	(0.98)			
(III vo)		(rere)		(0.97)			
(III ep)		(ep/ep)		(0.96)			
(III ah)		(Ah/ah)		(0.96)			
(III cd)		(Cd/cd)		(0.97)			
(III Da)		(Da/da)		(0.55)			
(III Dw)		(Dw/dw)		(0.95)			
(III vp)		(Vp/vp)		(0.95)			
III_c	Albino	cc AA E^dE^d mpmp	Subline of III	0.94	$a^3a^3b^4b^4c^7c^7$	4000	
X	Sooty yellow	$C(c^{ch2})$ aa ee bb Haha AsAs	Castle's small race	0.88	$a^3a^3b^4b^4c^7c^7$	2200	dk, c21, as
WH	Agouti	AA EE WHWH Lsls(5)	Rockefeller Inst. 1949. Crossed with strains X, III, etc.	0.72		2300	e, r^2, E^d
AC(3)	Black, recessive white marking	aa E E, du^ddu^d Acac(5), Sbsb(5)	Dutch	0.85	$a^3a^3b^5b^5c^7c^7$	2400	y, du^w, c^{21}
ACEP(3)	Blue eyed white	vv, epep	Dutch	0.92	$a^3a^3b^4b^4c^{21}c^{21}$	2400	hg,
ACCR (B)(3)(6)	Albino	cc sasa	Dutch	Inbred	$a^3a^3b\ b\ c^7\ c^7$	2400	y
(Y) (6)	Albino	cc sasa	Dutch	Inbred	$a^3a^3b^4b^4c^{21}c^{21}$	2400	c^7
OS	Black, minimal recessive white marking	aa E^dE^d, du^ddu^d, Osos(5)	Dutch Rockefeller Institute 1948	0.85	$a^2a^2b^4b^4c^7c^7$	3200	hydrocephaly(2)
AX	Chinchilla	$c^{ch3}c^{ch3}$ AA ww Axax(5)	Outcross of Chinchilla race V to strains III and X	0.68	$a^3a^3b^4b^4$	3500	du, bu, c^{ch2} c, hypospadias
AX_{bubu}	Albino	cc bubu	AX strain	0.76	$b^4b^4c^7c^7$	3500	c^{ch3}, c^{ch2}, a^1a
A	Albino	cc	Miscellaneous	0.95	$a^3a^3b^4b^4$	2700	l
C	agouti or black	AA or aa	Miscellaneous	0.98		2800	b, c, du

(APPENDIX CONT.)

Genes of importance to studies of constitutional disease being maintained in the colony are:

Achondroplasia (ac)
dach (Da) (6)
dwarf (Dw) (4)
renal cysts (rc)
mandibular prognathism (mp)
hypogonadia (hg)
lymphosarcoma (ls)
hemolytic anemia (ha)

angora (1)
furless (f)
rex^2 (r^2)
satin (sa)
adrenal hyperplasia (ah)
chondrodystrophy (cd)

ataxia (ax)
buphthalmia (bu)
epilepsy (audiogenic seizures, ep)
gamma globulin alleles, As^1, As^2, As^2
gamma globulin alleles, Ab^4, Ab^5
gamma globulin alleles, Ac^7, Ac^{7-}
osteopetrosis (os)
spina bifida (sb)

Lethal muscle contracture, hypognathia, splay leg, diminutive dwarf, and cleft palate occur sporadically in some strains and may be genetic.

For other gene symbols, see:

Sawin, P. B. 1955. Recent genetics of the domestic rabbit. Adv. Genet. 7:183-226.
Robinson, R. 1958. Genetic studies of the rabbit. Bibliog. Genet. 17:229-558.
Fox, R. R. 1974. Taxonomy and Genetics In Biology of The Laboratory Rabbit, p.1-22. S. W. Weisbroth, A. L. Kraus, R. E. Flatt (eds) Academic Press

Footnotes:

(1) Maximum coefficient in the strain computed according to Wright's formula for coefficient of inbreeding (F). Inbreeding is by sib mating or as close to sib mating as possible consonant with maintenance of the specific lethal or semi-lethal genes (indicated by underlining) and an optimal reproductive capacity and viability.

(2) Level of penetrance is dependant upon environmental conditions.

(3) Sublines of the same Dutch stock obtained from Rockefeller Institute in 1948.

(4) Formerly symbolized dw, then recognized in the heterozygote (Sawin 1955 Adv. Genet. 7:183) and the symbol changed to Dw to reflect a semidominant.

(5) The ac, Da, os, sb, ha, ls, ah, cd, and ax genes are maintained in their respective strains by progeny testing of prospective parents. Homozygous transmitters are obtainable from the same test.

(6) The ACCR (B) and ACCR (Y) strains are also referred to simply as strain B or Y respectively.

(7) Formerly da, now recognized, by ear papilla, in the heterozygote.

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